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13. ABSTRACT ***(Maximum 200 Words)***

Increased levels of ERK MAP kinases and increased MAP kinase activity have been demonstrated in malignant breast carcinomas. Therefore, regulators of MAP kinase activity are attractive targets for breast cancer therapeutic intervention. *Kinase Suppressor of Ras* (KSR) is a recently characterized component of a key signaling pathway that activates MAP kinases, the Ras/Raf/MAP kinase pathway. KSR appears to function as a scaffold protein, bringing together on a single platform many of the components involved in this signaling pathway for efficient signal transduction. KSR moves between different cellular compartments in response to extracellular growth signals. The phosphorylation state of KSR appears to play a significant role in determining its intracellular localization, which in turn affects its function. The goal of the current project is to identify specific kinases which bind to and phosphorylate KSR, thereby providing an opportunity to alter the phosphorylation state of KSR and its ability to function in the Ras/Raf/MAP kinase pathway. We have developed a method of purifying KSR-associated proteins which has yielded several candidate proteins that we are currently characterizing. These studies promise to yield valuable information regarding KSR function in breast cancer.

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Introduction

Kinase-Suppressor-of-Ras (KSR) is a recently characterized component of the Ras-Raf-MAP kinase signaling pathway (ref. 1-3). KSR is a phosphoprotein whose phosphorylation regulates its intracellular localization, which in turn may play a role in KSR physiology (ref. 4, 5). The objective of the project described herein is to identify kinases which phosphorylate KSR and to determine their role in KSR function, particularly their effect on the role of KSR in breast cancer cell signaling.

Body

Stated Task 1: Identify KSR-associated kinases (months 1-22)

We have generated several KSR fusion protein bacterial expression constructs (see figure 1). These constructs possess an amino-terminal GST or 6X histidine tag which allow binding of the fusion protein to either GSH Sepharose or nickel agarose, respectively. This solid phase affinity matrix can then be used for purification of KSR-binding proteins from mammalian cell extracts.

Figure 2 shows the results of expression of the constructs in E. coli followed by purification using either GSH Sepharose or nickel agarose. Expression level and relative purity of the constructs are analyzed using SDS-PAGE and Coomassie Blue staining. Our constructs expressed at varying levels, some of which were too low for use as an affinity reagent (data not shown). Figure 2 shows only the results for constructs which expressed at satisfactory levels. As can be seen by comparison to the BSA standards, the concentration of the fusion proteins on the agarose beads varies from below 0.1 mg/ml (His N539) to nearly 1 mg/ml (GST-CA4). We chose to use GST-CA4 as our affinity reagent in our early experiments because this fusion protein expressed at a higher level than other constructs. Moreover, CA4 contains serine 392, a phosphorylation site of interest to us since its phosphorylation regulates KSR intracellular localization (ref. 4). We reasoned that CA4 therefore should also contain the binding site for the kinase which phosphorylates serine 392, thus making GST-CA4 a suitable affinity reagent.

We next performed small scale pilot purifications of KSR binding proteins from HeLa cytosolic extract using GST-CA4 as affinity bait bound to GSH-Sepharose. We eluted bound HeLa proteins using GSH and analyzed the eluates by SDS-PAGE followed by silver staining, which provides much greater sensitivity than Coomassie Blue staining. As a negative control for the experiment, we passed HeLa extract over a GST GSH-Sepharose affinity column. A second negative control consisted of the GST-CA4 affinity column exposed only to buffer instead of cell extract. Figure 3 shows the results of one of our early experiments. In figure 3, three bands can be seen which bind to the GST-CA4 column but not to the control columns, suggesting that they specifically bind to CA4. Figure 4 shows a more recent experiment in which GST fusions of CA234, CA34 and CA4 were used as affinity reagent in separate columns. In this experiment, a number of potential KSR-binding proteins can be seen, three of which (indicated by arrows) appear to have been purified by each of the three KSR fusion proteins.

The next step in the process of identifying KSR-associated kinases involved scaling up the purification of KSR-binding HeLa cytosolic proteins approximately ten-fold in order to obtain sufficient amounts for analysis by MALDI-TOF mass spectrometry. The purification was performed in similar fashion as the small scale purification. Eluates were again analyzed by SDS-PAGE. In this case we used Coomassie Blue staining instead of silver staining to reveal bands of interest. Proteins that can be detected by Coomassie Blue staining should be present in sufficient amount for analysis by mass spectrometry. Figure 5 shows the results of a large scale affinity purification. The bands which we had seen in silver stained gels in the small scale experiments (figures 3 and 4) at approximately 72 kDa and 105 kDa were not prominent in this case. The reason for this is unclear, but is likely a technical issue that can be resolved. However, a protein at approximately 50 kDa (indicated in figure 5 by an arrow) appeared to bind to the GST-CA4 affinity column but not to the control columns. We therefore decided to have this protein analyzed by mass spectrometry.

To analyze the 50 kDa protein, we excised the band from the gel, along with the corresponding location in the two control lanes, and sent the gel slices to our collaborator, Dr. Natalie Ahn at the University of Colorado. Dr. Ahn has expertise in MALDI-TOF mass spectrometric analysis, as well as the necessary equipment to perform the procedure. Mass spectrometric analysis is performed on the fragments of the protein resulting from trypsin digestion, and determining the masses of these fragments yields a fingerprint which can be compared to a database to identify the protein. The analysis of our 50 kDa protein strongly suggested the identity of the protein to be EF1-gamma. EF-1 gamma is a recently characterized protein factor involved in protein translation in mammalian cells. EF-1 gamma is commonly overexpressed in colorectal and pancreatic cancers (ref. 6), but its function is unclear. EF1-gamma is not a kinase, however, and thus our search for a KSR binding kinase continues.

In the upcoming reporting period, we will continue to try to optimize the purification procedure using our GST-KSR fusion constructs as bait in order to identify additional candidates for mass spectrometric analysis. In addition, we will pursue an alternative approach which involves stable expression of KSR in HeLa cells, followed by large-scale growth of the cells and immunopurification of the KSR. Any proteins bound to the immunoprecipitated KSR can again be detected by the usual SDS-PAGE and silver staining procedures and analyzed by mas spectrometry. This approach will enable us to identify proteins which actually bind to KSR inside living cells, reducing the potential for misleading experimental artifacts. We have in fact already attempted to stably express KSR in HeLa cells, but have thus far met with technical difficulties which have prevented us from isolating viable stable clones. We believe we can overcome these problems, however, by modifying our experimental method.

Although this project focuses on identifying kinases which bind to KSR, KSR also binds other proteins which are not kinases but which are of interest to us nonetheless. Among proteins we found to interact with KSR in a yeast two-hybrid assay was a factor known as FHL-3 (unpublished observations). FHL-3 is a member of the FHL (four-and-a-half LIM

domain) family of proteins, and acts as a strong transcriptional coactivator of CREB (ref. 7). Since CREB has been observed to induce adipogenesis in 3T3-L1 preadipocytes (ref. 8), and we have found that deletion of KSR inhibits adipocyte differentiation (unpublished observations), we are currently exploring whether the interaction of KSR and FHL-3 may play a role in CREB-mediated transcriptional activation and induction of adipogenesis.

Stated Task 2: Determine role of KSR-associated kinases (months 18-36)

C-TAK1 (cdc25C-associated kinase 1) has recently been shown to bind KSR (ref. 5), and we have found that this kinase phosphorylates serine 392 in KSR, a site which is required for binding of KSR to 14-3-3. Furthermore, C-TAK1 appears to regulate the subcellular localization of KSR by controlling its ability to bind 14-3-3 (ref. 5), thereby controlling KSR function in MAP kinase activation. We are currently exploring further the effects of C-TAK1 on KSR intracellular localization and function.

Key Research Accomplishments

We have generated bacterial expression constructs for several KSR fusion proteins and have expressed and affinity-purified the proteins. We have used several of these KSR fusion proteins as affinity reagents to purify potential KSR-associated kinases from HeLa cytosolic extracts. Mass spectrometric analysis has identified one KSR-associated protein as EF1-gamma, a factor involved in protein translation which is overexpressed in gastrointestinal cancers.

Reportable Outcomes

The research has not yet progressed to the point of manuscript preparation or presentation at a research meeting.

Conclusions

The goal of the project described herein is to identify kinases which bind to KSR and to examine their effects on the function of KSR in the cell. KSR is a component of signaling via the Ras-Raf-MAP kinase pathway, the aberrant function of which appears to be involved in many types of cancer, including breast cancer (ref. 9, 10, 11). Identification of KSR-associated kinases may allow modulation of KSR phosphorylation and alter its role in MAP kinase signaling in breast cancer, with potential therapeutic benefits. To date, we have developed an approach which has significant potential for achieving the stated objectives. With further optimization of our procedures and development of complementary approaches, we anticipate that our efforts will yield valuable information which will help to clarify the function of KSR.

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Appendices

Figure 1: KSR expression constructs generated

Figure 2: Expression and purification of KSR fusion protein affinity constructs

Figure 3: Affinity purification of KSR-binding proteins from heLa cytosolic extract using GST-CA4

Figure 4: Affinity purification of KSR-binding proteins from HeLa cytosolic extract using GST fusions of CA234, CA34, and CA4.

Figure 5: Scaled-up affinity purification of KSR-binding proteins from HeLa cytosolic extract

Figure 1: KSR Expression Constructs Generated

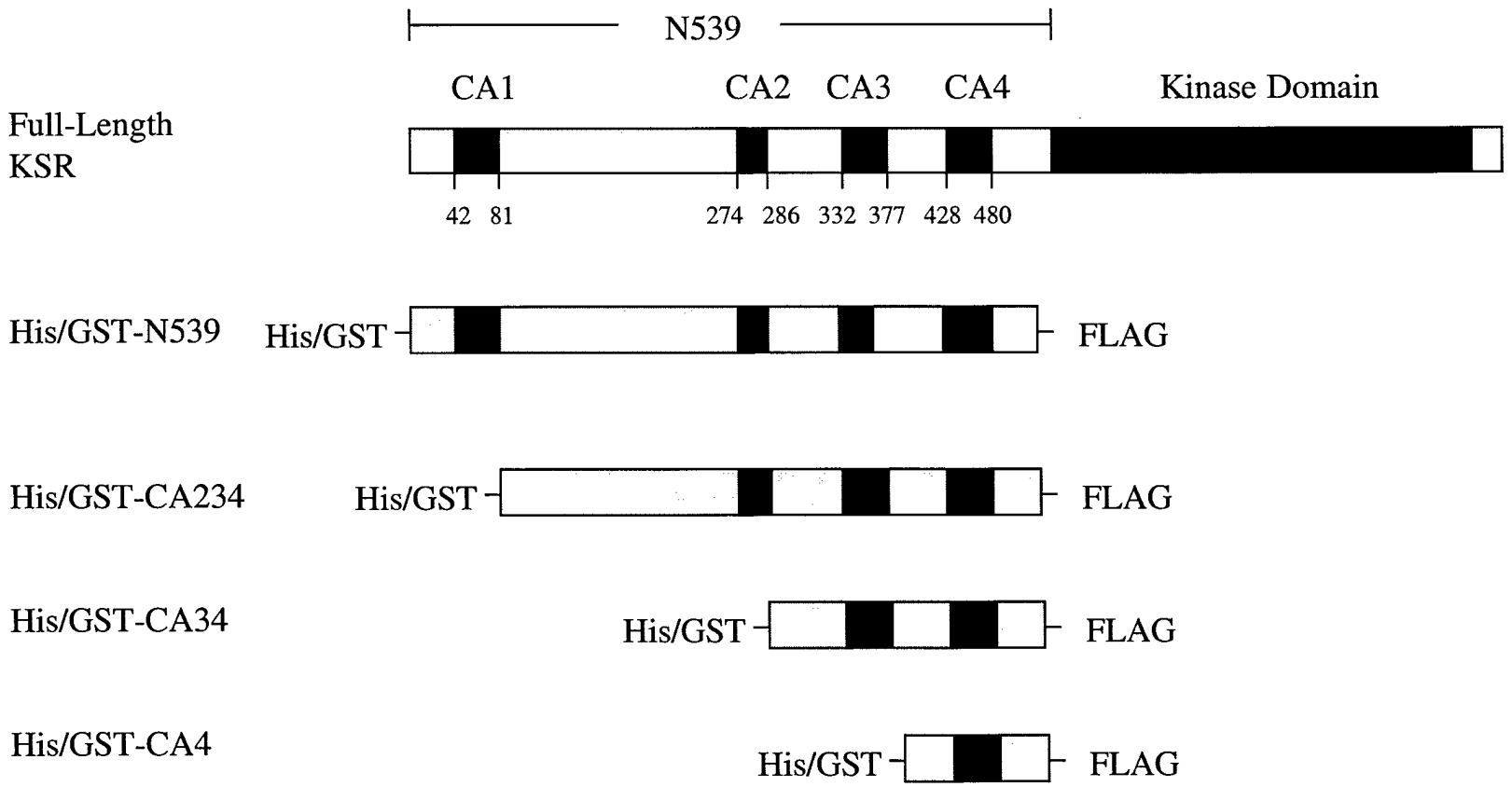

Figure 2: Expression and Purification of KSR Fusion Protein Affinity Constructs

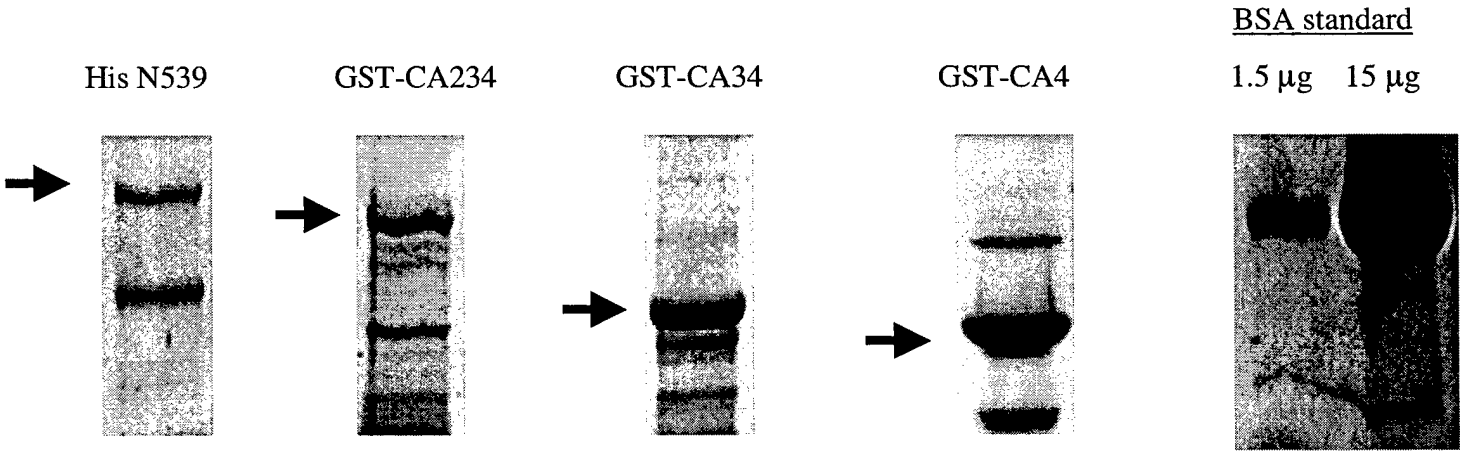

Figure 3: Affinity Purification of KSR-Binding Proteins from HeLa Cytosolic Extract Using GST-CA4

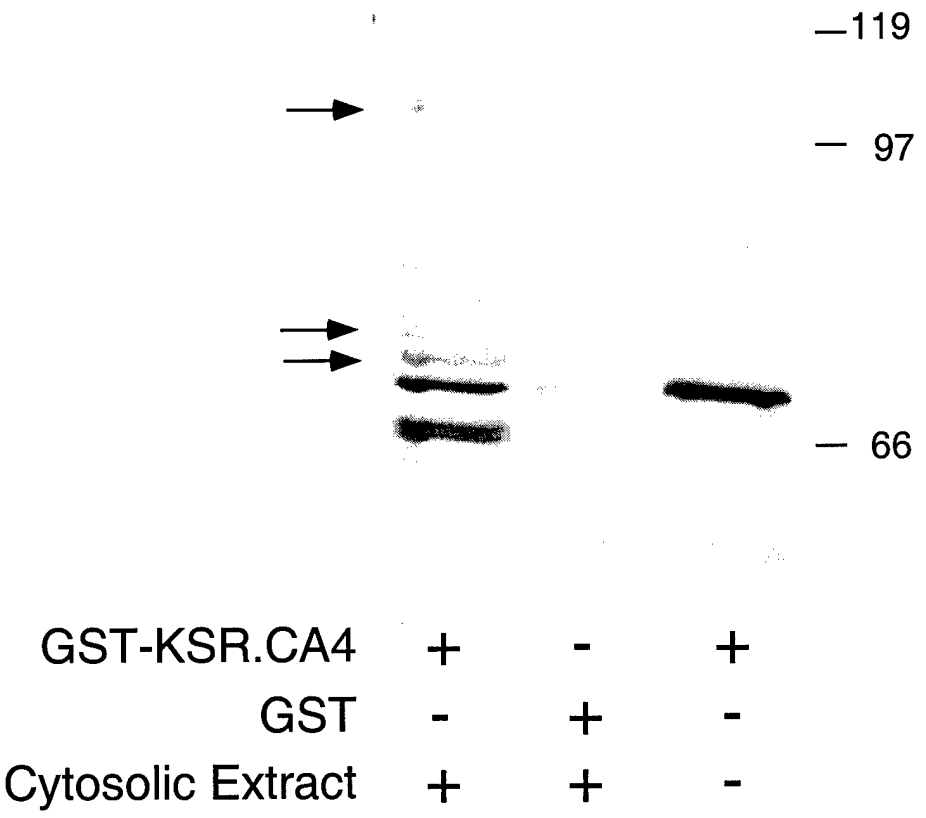

Figure 4: Affinity Purification of KSR-Binding Proteins from HeLa Cytosolic Extract Using GST Fusions of CA234, CA34, and CA4. Bands of interest are indicated by a dot next to the band, and arrows Indicate proteins which appear to be purified by all three constructs.

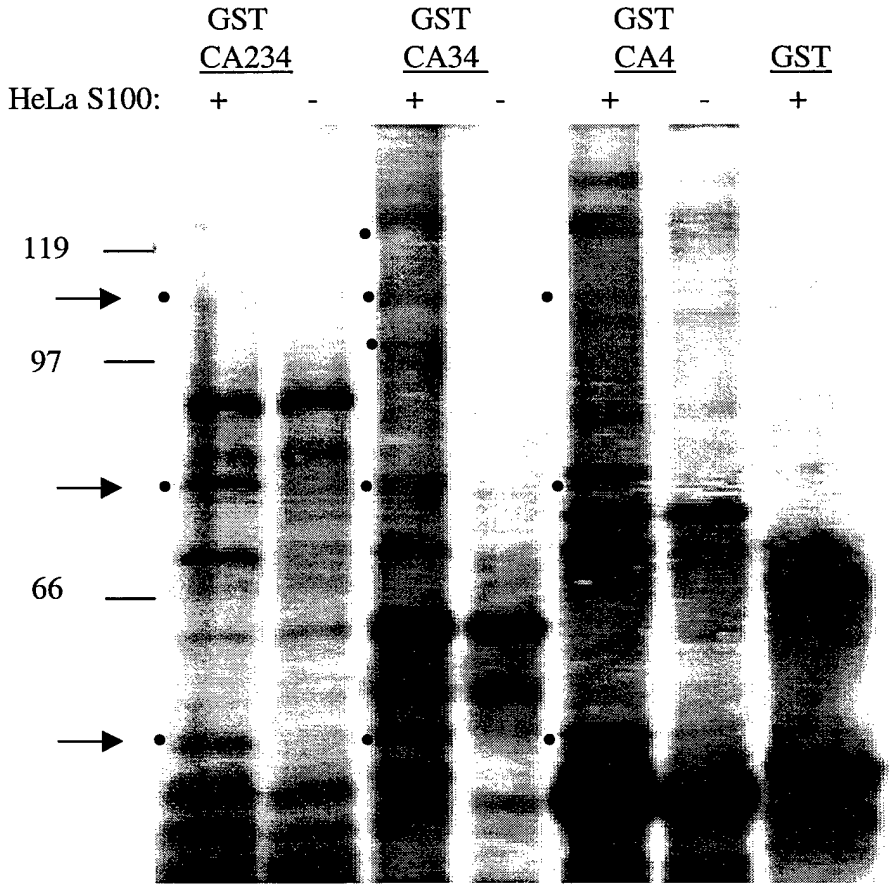

Figure 5: Scaled-up Affinity Purification of KSR-Binding Proteins from HeLa Cytosolic Extract